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A proteomics approach for the development of sarcoma biomarkers

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ABSTRACT

Sarcomas are rare and clinically diverse malignancies, and treatment optimization requires the development of suitable biomarkers. In earlier research employing proteomics analysis, we identified peroxiredoxin 2 as a biomarker of osteosarcoma (OS) that can predict response to neoadjuvant chemotherapy and verified its functional significance in the resistance of OS cells to chemotherapeutic drugs. In addition, in gastrointestinal stromal tumor (GIST), we identified pfetin as a prognostic biomarker and validated its prognostic utility in multi-institutional studies by immunohistochemistry. Here, we present an overview of our progress in sarcoma proteomics and discuss future perspectives.

1. Clinical features of sarcomas and the need for biomarker development

Sarcomas are rare and highly diverse mesenchymal malignancies, arising from bone, cartilage, muscle, fat, peripheral nerves, and adipose or fibrous connective tissues [1]. Sarcomas affect ~200,000 individuals worldwide each year, accounting for less than 1% of all adult malignant tumors. Sarcomas are classified into more than 50 histological subtypes, many of which have unique clinical, prognostic, and therapeutic features. Clinically, sarcomas range from curable tumors to those causing aggressive, incurable disease. Standard chemotherapy protocols have been established for only few sarcoma types; for others, numerous molecular-targeting treatments are currently under investigation [2]. In order to optimize the response of sarcomas to therapeutic intervention and minimize any treatment-related toxicity that could compromise clinical efficacy, novel biomarkers are urgently required.

2. Proteomics approach toward characterization of biomarkers using tumor tissues

The proteome is a functional representation of the genome that directly characterizes cell or organism phenotypes. Proteomics can provide unique proteome data on the level of protein expression [3,4]; status of protein complexes [5,6]; and post-translational modifications such as phosphorylation, ubiquitination, glycosylation, acetylation, and ribosylation [7,8], protein localization [9,10], and protein function [11,12]. By integrating these data with clinical observations, it may be possible to identify biomarkers that could be useful for evaluating the malignant potential of different sarcomas.

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For studying the sarcoma proteome, we had employed two-dimensional difference gel electrophoresis (2D-DIGE) [13], where protein samples labeled with different spectrally resolvable fluorescent dyes are separated by 2D gel electrophoresis (2D-GE). The gels are then laser-scanned using dye-specific excitation wavelength enabling to see each sample separately. In general, 2D-DIGE allows more comprehensive protein expression profiling than classical 2D-GE, which is especially important for the studies of cancer biomarkers [14–16]. First, gel-to-gel variations can be compensated for by using a common internal pooled control (a mixture of individual samples labeled with a fluorescent dye different from that of individual samples) allowing quantitative comparison of multiple specimens. Second, 2D-DIGE provides parallel assessment of several experiments, which is essential for biomarker studies based on repeated examination of many samples within a relatively short period of time. In 2D-DIGE, it can be achieved by simultaneous use of electrophoretic instrumentation, since the cost of performing electrophoresis is relatively low. Moreover, protein detection is performed using laser scanning, allowing high-throughput analysis of multiple samples. Third, contrary to conventional 2D-GE, in 2D-DIGE large-format gels displaying thousands of protein spots can be used [17,18] because, contrary to colorimetric staining, the detection by laser scanning does not require handling of fragile, easily breakable polyacrylamide gels. In our experiments, up to 5000 protein spots could be observed in a single gel during 2D-DIGE [16]. Finally, 2D-DIGE offers high sensitivity of protein detection [19], which is critical for biomarker studies based on size-limited clinical specimens. We have reported that very small samples (micrograms of protein), such as those obtained by laser microdissection, can generate thousands of protein spots after labeling with a highly sensitive fluorescent dye [19] (a detailed protocol published in [16]). Thus, the major advantages of 2D-DIGE as a modality for proteomic biomarker studies are comprehensive high throughput analysis, high sensitivity, and reduction of inter-gel variability, which provides quantitative comparison of biological changes and reduces experimental bias. The combination of these factors makes 2D-DIGE a more reliable approach to the identification of clinically relevant cancer biomarkers for prediction of treatment response compared to other proteomic techniques, which may be superior in terms of individual parameters. That is why 2D-DIGE has been widely used in biomarker studies resulting in identification of a number of proteins that correlate with clinical parameters in various cancers.

3. General strategy for biomarker development

In 2002, Anderson et al. published a landmark paper where they evaluated proteins according to the expected expression levels in plasma [20]. At that time, conventional proteomics made limited contribution to the list of plasma biomarkers used in the hospitals. Since then, the advances in proteomic technology resulted in considerable improvement of the sensitivity in protein detection, holding promise that comprehensive proteomics might instantly lead to successful biomarker identification. However, global expression profiling alone may not necessarily be so successful. Thus, transcriptomics, which quantitatively monitors mRNA synthesis using DNA microarray technology, has been extensively used in biomarker studies over the last decade [21–23]. However, among hundreds of reported mRNA candidate biomarkers, only few advanced to clinical application [24,25], suggesting that although comprehensive technology is potentially beneficial for biomarker discovery, it may not warrant successful identification of clinically relevant molecules.

Biomarker studies should be based on profound understanding of disease background and conducted to meet clinical demands [26,27]. Based on specific clinical requirements, samples should be appropriately stratified according to clinical characteristics, and informative proteins can be revealed through comparative studies. However, researchers conducting basic proteomic studies generally do not have medical background or access to clinical data. Therefore, interdisciplinary collaboration between basic and clinical scientists is mandatory in biomarker studies.

On the basis of this concept, in 2004, we launched a collaborative project on sarcoma proteomics that united efforts of basic and clinical researches. Six young medical doctors specializing in sarcoma have participated in our sarcoma proteomics project. In Japan, basic laboratory experience is a mandatory part of medical training, and employing a problem-oriented research style, we have incorporated clinical approach into our proteomics project.

The biobanking system at the National Cancer Center in Tokyo provides an invaluable source of clinical samples for research purposes. Tumor tissues (frozen in vapor nitrogen) from over 1000 sarcoma patients are stored anonymously but could still be traced by medical records such as pathological diagnosis, treatment, and clinical outcome. These samples have been the basis of our sarcoma proteomics project.

In addition to the proteome, we also used the data on the sarcoma transcriptome and genome and found that most of this information does not overlap and that integration of these data is quite challenging. These issues will be discussed elsewhere.

4. Approach to study sarcoma proteome

When we began sarcoma proteomic studies in 2004, there had been no established approaches to proteomics in cancer research, especially with regard to sarcoma. When we employed 2D-DIGE to generate global protein expression profiles of 80 soft-tissue sarcoma samples with seven different histological backgrounds, we found that histologically identical sarcomas shared common proteomic features [28], consistent with the results of a previous DNA microarray study showing a similar tendency for the sarcoma transcriptome [29].

Sarcomas exhibit a clinically wide spectrum from curative to malignant disease, the latter being associated with metastasis and treatment resistance. These clinical characteristics often correspond to histological subclasses and grading [30]. Accordingly, our preliminary observations encouraged further proteomic studies as part of general efforts in the discovery

of sarcoma biomarkers. We examined various sarcoma types, including osteosarcoma [31,32], Ewing's sarcoma [33–35], gastrointestinal stromal tumor [36–44], synovial sarcoma [45,46], myxoid liposarcoma, myxofibrosarcoma, leiomyosarcoma, alveolar soft tissue sarcoma [47], rhabdomyosarcoma, epitheloid sarcoma, and giant cell tumor. Here, we describe osteosarcoma (OS) and gastrointestinal stromal tumor (GIST) as examples of our sarcoma biomarker studies.

5. Osteosarcoma

Osteosarcoma (OS) is the most common type of primary bone cancer, which accounts for 35% of all primary bone malignancies [48] and frequently affects children and adolescents [49]. In Japan, approximately 200 patients develop OS annually. High-dose multi-agent neoadjuvant chemotherapy regimens have improved the cure rate of localized OS to approximately 80%, as compared with 15–20% achieved by surgery alone [50,51]. Neoadjuvant pre-operative chemotherapy targets micrometastasis and reduces tumor vascularity and growth [52], thus allowing complete eradication of all tumor cells after the surgery. In OS, the response to neoadjuvant treatment is evaluated in terms of post-treatment tumor necrosis; according to the HUVOS grading system, patients showing <90% necrosis are classified as poor responders [49]. Currently, OS response to induction chemotherapy following definitive surgery is the most reliable predictor of long-term outcome and is used to guide the choice of adjuvant chemotherapy [53–59]. In order to optimize therapeutic strategies and discover novel therapeutic targets, it is imperative to identify the molecular background of poor responders. Given that OS is a form of sarcoma with complex genetics [30], global expression studies may be effective for the identification of the proteins associated with clinical parameters. Thus, global transcriptome studies have identified several intriguing predictive biomarker candidates [60,61]; however, until recently their clinical utility has not been confirmed. In our proteomic studies, we attempted to clarify the molecular nature of OS resistance to neoadjuvant chemotherapy.

To develop biomarkers for the prediction of OS response to neoadjuvant treatment, we performed proteomics analysis of open (surgical) biopsy samples obtained from OS patients prior to induction chemotherapy. After pathologic diagnosis of OS, 12 patients received combination neoadjuvant chemotherapy with ifosfamide, doxorubicin, and cisplatin [31], and 13 were treated with methotrexate, doxorubicin, and cisplatin [32]. Patients were stratified for adjuvant therapy on the basis of their therapeutic response according to the HUVOS grading system at the time of surgery [49], and protein profiles of patients responsive and non-responsive to neoadjuvant treatment were compared using 2D-DIGE. Other than the response to treatment, no statistically significant difference in clinical parameters was observed. In the patients treated with ifosfamide, doxorubicin, and cisplatin, we identified 55 protein spots containing 38 unique proteins as predictive candidate biomarkers [31]. In the patients treated with methotrexate, doxorubicin, and cisplatin, 33 protein spots containing 27 unique proteins were determined as potential predictive biomarkers [32]. Further investigation revealed that the expression of peroxiredoxin 2 (PRDX2) was significantly upregulated in the patients with poor response to both types of combination neoadjuvant chemotherapy [31,32].

PRDX2 belongs to the peroxiredoxin family of antioxidant enzymes, important for hydroperoxide detoxification and highly reactive with hydrogen peroxide, organic hydroperoxides, and peroxynitrite [62]. PRDX2 scavenges free radicals, and is suggested to protect tumor cells from environmental stress such as chemotherapy drugs and to promote tumor formation. Thus, PRDX2 has been associated with the malignant potential of breast cancer [63], colorectal cancer [64,65], prostate cancer [66], and melanoma [67]; however, until our studies, its role in OS development was unclear. PRDX2 is known to promote proliferation [64], metastasis [67], stress response [63,65], resistance to therapy [68], and signal transduction [64,66] in tumor cells, and is considered to be a potential drug target for anticancer therapy [69,70]. Using western blotting, we confirmed that overexpression of PRDX2 was associated with the resistance of OS patients to neoadjuvant chemotherapy. Among the patients who received ifosfamide, doxorubicin, and cisplatin, independent cases were examined, and the predictive value of PRDX2 was successfully validated [31]. In a prospective study, we found that one patient who had a PRDX2-positive primary tumor showed poor response to treatment with ifosfamide, doxorubicin, and cisplatin. We also confirmed PRDX2 localization in OS cells by immunohistochemistry [32]. Using gene silencing assays, we functionally verified PRDX2 contribution to both resistance to individual chemotherapeutic drugs such as methotrexate, doxorubicin, and cisplatin, and malignant potential in three OS cell lines. When PRDX2 expression was diminished by specific siRNAs, OS cells acquired sensitivity to chemotherapeutic drugs and showed a reduced potential for cell proliferation, invasion, and migration [32]. We submitted a patent application for the predictive utility of PRDX2 in OS and started a nationwide multi-institutional validation study.

In this study, the use of open biopsy samples obtained prior to chemotherapy was a critical factor. Because most OS patients receive pre-operational neoadjuvant chemotherapy, the affected surgically resected tissues become necrotic and thus may not be adequate resources for the discovery of predictive OS biomarkers. On the contrary, open biopsy samples routinely obtained from OS patients for pathological diagnosis present a valuable source of clinically relevant novel biomarkers. However, the open biopsy samples are rarely stored as frozen or formalin-fixed and paraffin-embedded (FFPE) tissues, limiting the availability of clinical specimens for biomarker development. To compensate for this limitation, we verified the functional significance of PRDX2 protein expression in vitro using OS cell lines. In general, the shortage of clinical material is the main problem for the studies of rare malignancies such as sarcoma, and the only workable approach may be functional verification and independent validation performed systematically over a long period of time.

6. Gastrointestinal stromal tumor

Gastrointestinal stromal tumor (GIST) is the most common primary sarcoma of the gastrointestinal tract, representing

1–3% of gastrointestinal tumors and affecting 15–20 in 1,000,000 people [71,72]. GIST cells originate from the interstitial cells of Cajal, the pacemakers of peristaltic contraction [73], and are characterized by unique genetic mutations in the c-*kit* or *PDGFRA* genes [74–76]. The clinical course of GISTs spans a wide spectrum ranging from a curable disorder to a highly malignant disease. Treatment of GIST patients harboring c-*kit* or *PDGFRA* mutations, with tyrosine kinase inhibitors such as imatinib and sunitinib, has been proven to be effective in cases of inoperable or metastatic tumors. As adjuvant imatinib treatment significantly improves recurrence-free survival [77], risk stratification for imatinib therapy has become an important step in the care of GIST patients. Three clinical features of primary GISTs are used for risk assessment: size, mitotic index, and location [74,78]; however, a subset of GIST patients classified as low-risk occasionally has metastasis. Therefore, in order to increase the specificity and sensitivity of risk stratification for imatinib treatment, it is necessary to search for genetic abnormalities other than *c-kit* and *PDGRFA* that are associated with GIST progression. These include aberrant expression of ezrin [79], raf kinase inhibitor protein [80], COX-2 [81], Bcl-2, carbonic anhydrase II [82], cell cycle/apoptosis regulators [83], and DNA methylation of specific tumor suppressor genes. Global mRNA expression studies conducted in GIST patients have identified various potential biomarker candidates [84,85]; however, these genetic aberrations do not completely explain the mechanisms of GIST aggressiveness, and no standard protocols based on these genetic events have yet been established.

To identify metastasis-associated proteins and develop prognostic biomarkers, we examined surgically resected tumor tissues from 17 GIST patients with different post-surgical outcomes: 8 patients had metastasis at diagnosis or developed it within 1 year after surgery, and 9 did not develop metastasis during 43–88 months of the observation period. These populations were classified, respectively, as high-risk and intermediate/low-risk groups based on pathological observations; none of the patients received adjuvant therapy. Using 2D-DIGE, we found 43 protein spots containing 25 unique proteins that showed significant intensity differences between the two groups. Among these proteins, pfetin detected in 8 protein spots was significantly associated with favorable outcome; this association was confirmed by western blotting and immunohistochemistry. In addition, quantitative RT-PCR analysis also showed that the expression of pfetin mRNA was significantly different between the two patient groups, although to a lesser degree than that of the protein [36].

Pfetin was originally discovered the fetal cochlea [86], consistent with the neuronal origin of GIST in the gut. Pfetin has a potassium channel-like structure and is involved in the GABA(B) receptor complex [87]. Although aberrant regulation of potassium channels has been associated with the malignant potential of tumor cells [88] and considered as candidate treatment target, until our study, the association of pfetin expression with favorable prognosis in GIST was never demonstrated.

We then confirmed our findings by immunohistochemistry, which is widely employed in clinical validation studies. Using an original polyclonal pfetin antibody [86], we were able to confirm the prognostic utility of pfetin in 210 GIST cases: the 5-year disease-free survival rate of GIST patients with pfetin-positive primary tumors was 93.9%, whereas that of patients with pfetin-negative tumors was 36.2% ($P < 0.0001$). [36] We further validated the prognostic utility of pfetin in additional 100 [38], 40 [89], and 72 [40] GIST cases at three hospitals by immunohistochemistry using monoclonal antibodies against recombinant pfetin developed in our laboratory. In total, we examined the prognostic value of pfetin detection by immunohistochemistry in 422 GIST cases. Our results clearly demonstrated that pfetin was an independent prognostic factor, also applicable for risk classification. When we stratified GIST patients, we found that pfetin had prognostic value in each risk classification group. Later, Hasegawa et al. in a study of 64 GIST patients in two hospitals obtained similar results [43].

We discovered pfetin using 2D-DIGE in 2005, and reported its prognostic utility after validation in 210 GIST cases in 2008 [36]. Over the next few years, we performed an additional validation study on 212 GIST cases [38,40,89] and collaborated with other researchers in a study involving 64 patients [43]. In 2014, our original monoclonal antibody against pfetin was commercialized (MBL, Japan) [44], and we are now planning a nationwide clinical study of pfetin as a prognostic biomarker in GIST. Our experience indicates that the discovery of a cancer protein biomarker could be accomplished in a relatively short period, but clinical validation took considerably longer time. It seems likely that a similar timetable would also apply to biomarker studies in other malignancies, particularly rare ones.

In addition to pfetin, 2D-DIGE-based proteomic analysis resulted in the discovery of other promising proteins associated with different post-operational outcomes for GIST patients [39]. Among these proteins, ATP-dependent RNA helicase DDX39 was confirmed by immunohistochemistry to be significantly associated with poor prognosis in 72 GIST cases [39]. As DDX39 and pfetin showed opposite expression patterns, the combination of these two GIST biomarkers potentially increases the probability of correct diagnosis [40]. Recently, global expression studies have identified ETV1 as a transcription factor unique to GIST cells and have shown that ETV1 and the mutated *c-kit* promoted tumor growth in a coordinated manner [90]. However, we found that ETV1 did not have prognostic utility because there was no correlation between clinical outcome and ETV1 expression in GIST patients [42], confirming the results of a previous study [91]. Gene silencing experiments have demonstrated that KCTD10, a potassium channel tetramerization domain (KCTD)-containing protein, is regulated by ETV1, suggesting the association of KCTD10 expression with the malignant phenotype of GIST cells [90]. We tested this hypothesis by examining KCTD10 expression using immunohistochemistry, and found, contrary to our expectations, that it was correlated with a favorable GIST prognosis [42]. These data suggest that a number of potential biomarker candidates with different prognostic utility in GIST can be successfully identified by proteomics approaches.

7. Perspectives

The rarity of sarcoma limits the number of clinical samples available for biomarker development. There are several approaches to circumvent this problem. For example, FFPE tissues are suitable for long-term storage and have been successfully used for proteomic analysis in cancer research. However, the number of FFPE sarcoma samples remains insufficient to solve the problem. It is also difficult to establish national or international collaborative efforts in order to share valuable clinical material in the early discovery phase. Moreover, as the pathological diagnosis of sarcomas is sometimes controversial, and standard treatments are not always available, the integration of samples and data from different institutions may give rise to confusing results. At present, there is no instant solution to the limited number of sarcoma samples. However, the following approaches may be worth considering in sarcoma biomarker studies.

Thorough functional verification may be of help in biomarker discovery. Genes that have a significant association with clinical observations are likely to be involved in the biological mechanisms underlying poor prognosis and resistance to treatment and may have prognostic value. Based on this hypothesis, PRDX2 has been identified as a candidate prognostic biomarker in OS; however, a functionally important protein ETV1 has not consistently demonstrated its prognostic utility [42]. As functionally relevant genes do not always have clinical value, mechanistic data indicate, but do not prove, clinical significance of candidate biomarker genes prior to validation studies. There is another problem for functional studies conducted in vitro: cell lines are available for only limited sarcoma histological types. Therefore, to facilitate biomarker development by functional studies, novel type-specific sarcoma cell lines should be established and shared in the research community.

Consideration of molecular mechanisms involved in sarcoma development may be another approach to compensate for the limited number of clinical samples. For example, GISTs may have a relatively homogeneous molecular background, owing to shared genetic alterations in the *c-kit* or *PDGFRA* genes. Thus, we started our biomarker discovery program using only 17 GIST cases and identified pfetin, which was subsequently validated for prognostic utility in approximately 500 cases at six hospitals. The successful characterization and clinical application of pfetin may be attributable to a relatively homogeneous molecular background underlying GIST progression. On the other hand, only a few GIST-related proteins discovered using 2D-DIGE were successfully validated by immunohistochemistry, suggesting that common mechanisms of carcinogenesis may not always warrant successful biomarker discovery. The classification of sarcomas based on global gene expression profiling may contribute to the molecular background-focusing approach, and mRNA and protein global expression studies should be extended [28,29].

Focusing on technical characteristics of proteomics methods may be an effective strategy. Pfetin was detected in 8 protein spots showing higher intensity in patients with favorable disease outcome. In 2D-DIGE, single gene products can appear in multiple protein spots, probably as a result of post-translational modifications, and the intensity of such spots does not always correspond to protein expression level measured by SDS-PAGE/western blotting [34,92,93]; expression data obtained by mass spectrometry may have the same problem. In such cases, the results of subsequent immunohistochemical analysis may be discordant with those of proteomics. For further immunohistochemical validation, we may need to select proteins present in a single spot or those detected in the protein spots showing similar intensities within the same sample group; the latter may be considered as multiple biomarkers measurable by single antibodies. However, in proteomics studies based on 2D-DIGE, only protein spots showing significant intensity differences between sample groups are subjected to protein identification by mass-spectrometry, and the number of protein spots containing target proteins remains unknown. Accordingly, this strategy is not always applicable for protein selection.

In conclusion, our experience suggests that a multi-dimensional approach should be employed for the biomarker development in rare cancers such as sarcomas. The strategy based on reasonable sample stratification according to cancer biology and selection of promising proteins for subsequent validation according to their functional significance together with appropriate proteomic techniques may increase the chances of successful biomarker identification. Moreover, it is not possible for a single research group to conduct all the studies required for identification and validation of sarcoma biomarker candidates; inter-disciplinary and multi-institutional collaborations are essential. Although the strategy described in this article has been focused on sarcomas, it can be generally applied to other rare malignancies.

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Conflict of interest

There are no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.06.004.

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